EXHIBIT C

REVIEW

HLA Class II Tetramers

Tools for Direct Analysis of Antigen-Specific CD4+ T Cells

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Introduction

CD4+ T lymphocytes recognize peptide antigens presented by major histocompatibility complex (MHC) class II molecules, and are central to immune activation and regulation. This pivotal role is evident both in immune responses to pathogens and in the initiation of autoimmunity, although antigen-specific CD4+ T cells exist at very low frequencies in patient peripheral blood, frequently in the range of 1:6,000 to 1:100,000. To identify and analyze these cells, one must traditionally use functional assays, such as antigen-induced proliferation or cytokine secretion, which infer T cell specificity from semiquantitative or qualitative outcome measures. New techniques using MHC tetramers can now be added to the immunologic toolbox to expand these types of assays, improving sensitivity and quantitation.

MHC tetramers are multimeric forms of soluble recombinant HLA molecules associated with specific bound peptide antigens. As illustrated in Figure 1, the recombinant MHC molecules are assembled into multimers to provide multiple ligands for enhanced interaction with antigen-specific T cell receptors (TCRs). These multimers are surrogates for the MHC–peptide ligand that is expressed on the surface of an antigen-presenting cell in a normal immune response. Individual MHC–

TCR contacts are low-affinity interactions (1); the multiple contacts inherent in the tetramer interaction increase the overall avidity, an important element in the detection and analysis of the MHC–TCR interaction detected with tetramers.

The CD4+ lymphocyte, which is a key contributor to immune activation, helper function, cytokine secretion, and immune regulation, recognizes the MHC–peptide complex, using its antigen-specific TCR for target specificity. Whereas CD8+ T cells are directed toward class I–peptide complexes, CD4+ T cells preferentially recognize MHC class II–peptide complexes. By binding to the TCR, a labeled tetramer molecule becomes a specific marker, identifying T cells by virtue of their antigen specificity.

Class I MHC tetramers

Originally described in 1996 (2), MHC class I tetramers have become widely used for quantitation of antigen-specific CD8+ T cell responses. MHC class I tetramers contain HLA–A or HLA–B molecules assembled with peptides from pathogen-associated or cellular targets, and have been used to enumerate CD8+ T cells to those targets, as well as to study CD8+ T cell specificity, avidity, and phenotype. For example, whereas tetramer analysis detected chlamydial epitope-specific CD8+ T cells in the peripheral blood of infected individuals at frequencies up to 0.2% (3), a similar analysis using Epstein-Barr virus–specific tetramers found the primary response in infectious mononucleosis blood to include up to 5% of the CD8+ T cells specific for 1 viral epitope (4). In patients with cytomegalovirus reactions posttransplantation, tetramer analysis of the peripheral blood identified up to 12% of CD8+ T cells (5). Thus, the range of antigen-specific cells detected by tetramers was enormous, and varied depending both on
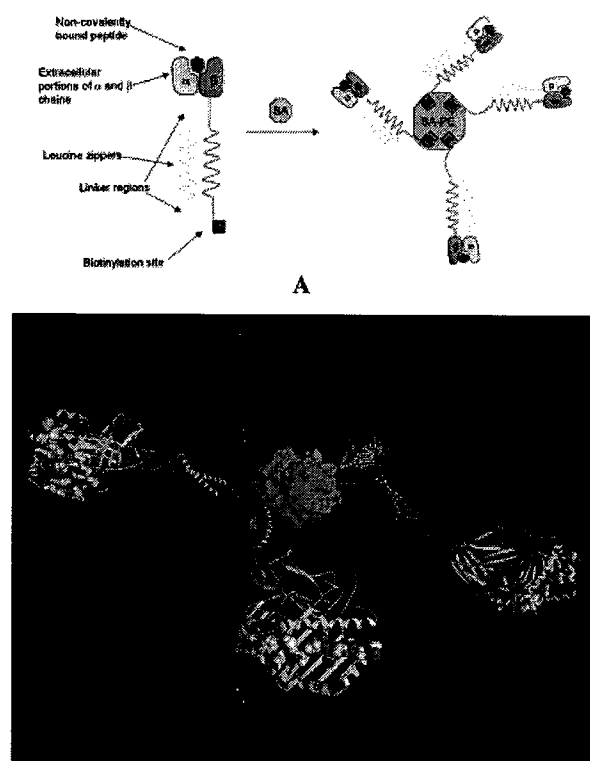
Figure 1. HLA class II tetramers. Soluble recombinant major histocompatibility complex (MHC) class II chains are assembled as biotinylated α/β dimers using leucine zipper and linker sequences. **A,** Schematic version of the structure, which is tetramerized via its interaction with streptavidin (SA). **B,** Molecular model of the complete tetramer; streptavidin (in blue) anchors long, flexible linker arms, which terminate in the MHC–peptide interfaces recognized by the antigen-specific T cell receptor. PE = phycoerythrin.

the pathogen and on the clinical setting. Indeed, fluctuating levels of tetramer-positive CD8+ T cells can be used as a clinical correlate of disease activity in chronic hepatitis (6) or to enumerate expansion of antigen-specific cells after vaccination against hepatitis B (7).

When coupled with methods that predict peptide binding to MHC class I molecules, tetramer analysis can be extremely useful for identifying T cell epitopes; for example, studies of CD8+ T cell responses in *Mycobacterium tuberculosis*-infected individuals have identified dominant epitopes restricted by both HLA–A2 (8) and HLA–B35 (9), simply by demonstrating the presence in blood of cells that bound the appropriate MHC–peptide tetramer complex.

The application of class I tetramers to study self antigens has been most extensively developed in studies of tumor antigens. For example, peptides from

melanoma-associated antigens loaded into HLA–A2 tetramers have been used for patient monitoring, phenotyping, and clinical correlations in patients with melanoma and in cancer vaccine trials (10–14). Insights based on the strength of tetramer binding have been used to infer the overall avidity of the TCR–MHC–peptide recognition complex, information that may help direct the design and use of candidate vaccines for inducing tumor-specific immunity (15–17).

Class II MHC tetramers

In contrast to these successful examples of the use of class I tetramers, success with MHC class II tetramers has lagged. Structural differences between class I and class II molecules necessitate different approaches to tetramer design and use. The single-chain class I molecule can be efficiently renatured in the presence of antigenic peptides to yield a stable β_2-microglobulin–associated class I monomer (18,19); engineering a soluble form carrying a biotinylation tag allows tetramerization with avidin (1). Class II molecules are noncovalent dimers of α and β chains, which have a variable range of stability and solubility in solution. Peptides bind into the α/β chain groove of class II molecules based on specific interactions with amino acid side chains, which confer specificity, determining which peptides will bind which class II molecules. Detection of individual CD4+ T cells based on their antigen specificity has been hampered by 2 significant barriers: the low frequency of such T cells in human peripheral blood and the low avidity of binding between the TCR and the MHC–peptide complex.

Different approaches to producing class II tetramers, to circumvent these problems, have been tried. One is the covalent synthesis of single-chain class II–peptide complexes, directed by engineering peptide-specific complementary DNA (cDNA) sequences proximal to the β chain cDNA (20). In this strategy, the resulting polypeptide refolds with the peptide sequence extended from the amino terminus of the class II molecule. A tethering linker sequence in the peptide allows enough flexibility for the peptide to occupy the peptide binding groove in the mature class II molecule. A presumed advantage of this methodology is that every class II molecule produced contains the same peptide sequence; a potential disadvantage is that a separate molecular construct must be produced for every class II–peptide tetramer designed.

The second approach that has been used is to load the biotinylated class II α/β dimer with exogenous

peptides, followed by multimerization with avidin (21). The principal advantage of this technique is that a single MHC molecule can be loaded with a large number of distinct peptides, greatly expanding the number of T cell epitopes that can be studied. Investigators at our laboratory have used this method for analysis of a variety of T cell responses, ranging from viral to autoimmune specificities. Strategies to quantitate and characterize the T cell response, and to use this technology for monitoring the status of patients with immune disorders, are rapidly evolving, as detailed below.

Figure 1 illustrates the configuration of the HLA class II tetramers used in our studies (21–25). As shown in Figure 1A, recombinant class II molecules are produced that incorporate "leucine zipper" motifs, replacing the native transmembrane and cytoplasmic portion of the molecules. The addition of these leucine zipper sequences provides both stability and solubility to the complex, which is produced in stably transfected *Drosophila* cells and subsequently purified by affinity chromatography (21). One chain incorporates a site for enzymatic addition of biotin, enabling construction of the tetrameric complex by the addition of streptavidin, which has 4 biotin binding sites.

Figure 1B shows a molecular model of the resulting class II tetramer. Each of the 4 termini contains a class II–peptide interface suitable for binding to antigen-specific TCRs. Flexible linkers on either side of the leucine zipper region provide considerable structural flexibility, which likely allows (and perhaps facilitates) some movement in clustering of the MHC–peptide–TCR complex when tetramers contact the surface of an appropriate antigen-specific T cell.

By using fluorophore-labeled streptavidin molecules, T cells that bind specific tetramers can be readily distinguished from those that do not, using flow cytometry. Examples are shown in Figure 2, using an HLA–DR4–HA tetramer, where the hemagglutinin (HA) antigen is an immunodominant peptide epitope (residues 307–319) from the hemagglutinin protein of the influenza A virus. Figure 2A shows the flow cytometry analysis for CD4+ cells derived from the lymph nodes of mice transgenic for HLA–DR4, which have been immunized with the HA peptide. A small number of cells are identified by fluorescence flow cytometry, indicating antigen-specific tetramer binding, although the number of cells identified is very low.

Figure 2B shows similar identification of human HA-specific CD4+ T cells in an individual 3 weeks following vaccination with the influenza vaccine. The left side (day 0) shows tetramer staining of lymphocytes
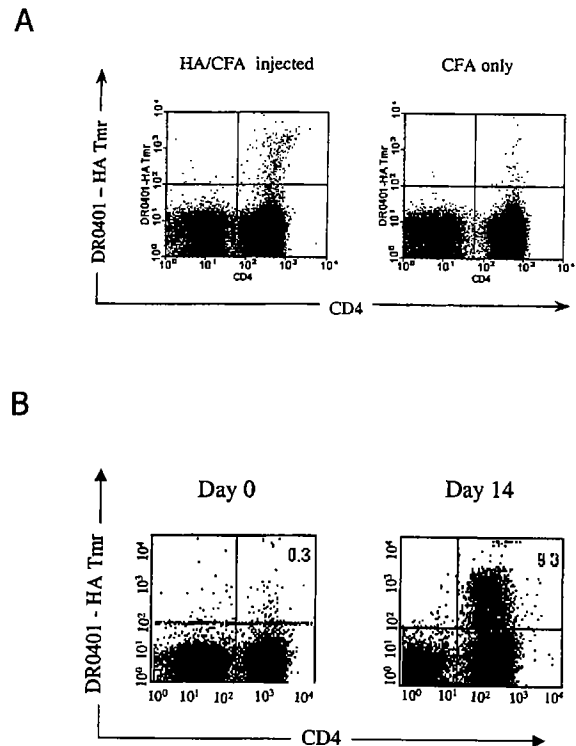

Figure 2. Detection of tetramer-positive antigen-specific CD4+ T lymphocytes. A, Murine and B, human lymphocytes analyzed by flow cytometry using HLA–DR4 tetramers loaded with an immunodominant hemagglutinin (HA) peptide from influenza hemagglutinin. A, Mice transgenic for HLA–DR4 were immunized either with HA in Freund's complete adjuvant (CFA) or with adjuvant alone, and the primary draining lymph node cells were analyzed by flow cytometry after 6 days. B, Peripheral blood lymphocytes from an individual immunized with influenza vaccine were studied before (day 0) and after (day 14) in vitro expansion with specific HA peptide antigen. The percent of tetramer-positive CD4+ cells relative to the total population is indicated in the upper right quadrants.

directly from the peripheral blood, while the right side (day 14) shows tetramer staining after 2 weeks of in vitro expansion with antigen-presenting cells and HA peptide. The large expansion of tetramer-positive antigen-specific cells (from 0.3% to 9.3% of total cells) represents both the accumulation of proliferating antigen-specific cells and the loss of unrelated T cells during in vitro culture. The low number of antigen-specific CD4+ cells in peripheral blood poses challenges for the sensitivity of flow cytometry; it is difficult to detect cells at frequencies below 0.2% (1:500) since background staining using tetramer loaded with an irrelevant, nonspecific peptide may approach 0.1% in these types of assays. However, as shown in Figure 2, antigen-specific T cells

.41%   **0.04%**

DR0401 – HA –tetramer PE

2.6%   **96.9%**
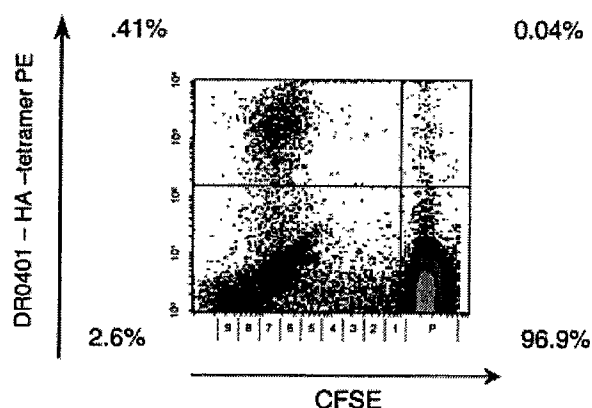
CFSE

Precursor Frequency .0041/2^7 = **1** in **30,000**

Figure 3. Estimation of precursor frequency of CD4+ tetramer-binding cells by tetramer analysis. Lymphocytes were labeled with CFSE prior to ex vivo expansion in the presence of specific antigen; the undivided, antigen-nonresponsive cells in the lower right quadrant represent 96.9% of the lymphocyte population, while 0.41% (in the upper left quadrant) are tetramer-positive and demonstrate proliferation by dilution of the CFSE dye. Calibration of the CFSE dye dilution axis indicates that these tetramer-positive cells have divided an average of 7 times, yielding a calculated precursor frequency of 1/30,000. HA = hemagglutinin; PE = phycoerythrin. Adapted, with permission, from ref. 21.

can readily be identified after in vitro activation and expansion.

Therefore, we developed a combined technique to determine the frequency of antigen-specific cells in peripheral blood, in which cell divisions and tetramer staining are determined simultaneously (21). In this system, peripheral blood lymphocytes (PBLs) are labeled with CFSE, a fluorescent dye that stably binds and is retained intracellularly (26). Cells are then incubated with antigen-presenting cells and specific antigen and cultured in vitro for 4–7 days. During this time, dividing cells partition the fluorescent CFSE dye with each cell division. Cells are stained with tetramer prior to analysis, and separate fluorescent wavelengths are used to detect tetramer binding and CFSE dye fluorescence simultaneously.

As shown in Figure 3, this method allows for the straightforward calculation of precursor frequencies for cells in peripheral blood that respond to specific antigens. This approach is particularly useful when, as in the example in Figure 3, the frequency of such antigen-specific cells in peripheral blood is exceedingly low,

below the threshold for detection in the unamplified sample. This requirement for in vitro amplification prior to tetramer detection poses some problems for analysis. Changes in T cell phenotype, differing rates of cell death, and selected expansion of higher-avidity cells are likely to occur during culture, potentially influencing quantitation of the tetramer-positive cells.

Nevertheless, it is likely that some sort of in vitro amplification is necessary for routine clinical use, since direct detection of antigen-specific cells, both in peripheral blood and in synovial tissue, has so far yielded very low or absent tetramer-binding populations (27,28). In studies of T cell responses to the *Borrelia*-associated outer surface protein A (OspA) antigen in patients with Lyme arthritis, Meyer et al observed very low frequencies of tetramer-positive cells in peripheral blood, with only 1 of 6 patients showing levels of specific tetramer staining >0.05%. Notably, in 2 of 6 patients this level of staining was achieved with control tetramer. Nonetheless, Meyer and colleagues were able to successfully obtain OspA-specific T cell clones from peripheral blood by sorting on this very small population. In addition, 1 patient had high levels (3%) of tetramer-positive cells staining with OspA tetramer in synovial fluid (27). In the other reported study using synovial fluid, neither DR4–GP39 nor DR4–collagen peptide tetramers showed significant staining of rheumatoid arthritis (RA) synovial cells, consistent with the interpretation that such cells were either absent or present at very low frequency. Alternatively, it is possible that the specific peptide–DR4 complexes used were not representative of the major T cell specificities in the synovial fluid samples (28).

Fluorescence flow cytometry uses a range of wavelengths compatible with multiple fluorescent markers. By performing simultaneous analysis of tetramer fluorescence with other T cell parameters, information about T cell specificity and phenotype can be directly gauged, even within very complex samples. Examples are shown in Figure 4, in which tetramer analysis is combined with fluorescent markers for TCR use or for cytokine secretion to characterize the antigen-specific T cells. In Figure 4A, PBLs were stained with antibodies to specific TCRβ chains before and after in vitro expansion with the flu HA peptide. CD4+ T cells from blood express most of the V$_\beta$ TCR specificities (staining for V$_\beta$2 and V$_\beta$3 families is shown for illustrative purposes). After in vitro expansion with HA antigen, the tetramer-positive cells show a strong preference for V$_\beta$3 staining, whereas none is V$_\beta$2+. Using a panel of anti-V$_\beta$ antibodies in this experiment, both V$_\beta$3 and V$_\beta$13 showed
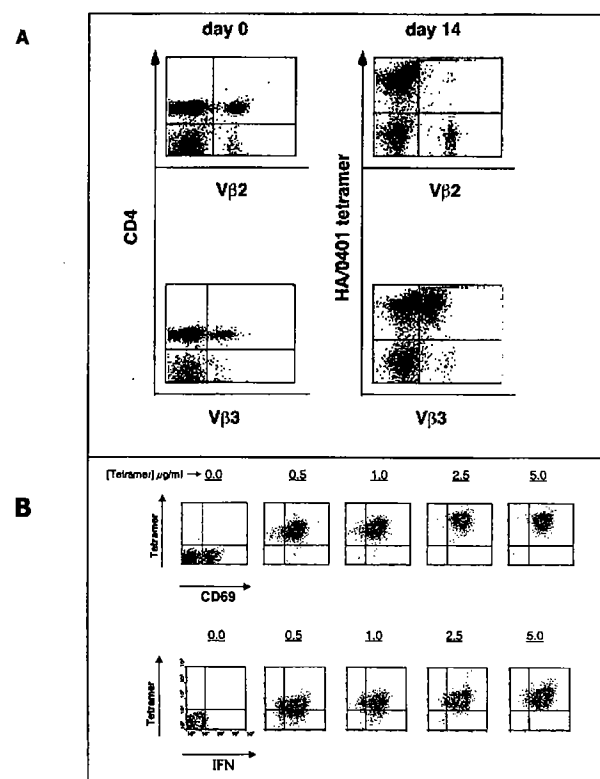
Figure 4. Multiparameter analysis of tetramer-positive CD4+ lymphocytes. Clonal T cell markers (**A**) or activation markers (**B**) can be assessed directly on tetramer-positive cells using flow cytometry. The biased expression of the Vβ3 T cell receptor molecule among T cells responding to the HA epitope is shown in A; lymphocytes were stained with antibodies to specific V$_\beta$ T cell receptor elements either directly (day 0) or after expansion with antigen (day 14). Simultaneous tetramer staining and acquisition of activation markers, either CD69 or interferon-γ (IFN) production, on a clonal population of CD4+ lymphocytes stimulated with specific major histocompatibility complex–peptide complexes is documented in **B**.

expansion in the tetramer-positive compartment, consistent with an oligoclonal V$_\beta$ preference for T cells reacting to this antigen. Indeed, it has previously been described in conventional T cell cloning experiments that the predominant T cell response to influence HA is indeed associated with the preferential use of the V$_\beta$3 TCR (29).

Figure 4B shows a different kind of T cell phenotyping, in which cellular response profiles by tetramer-positive T cells are evaluated by flow cytometry. In the sample shown, both activation markers, CD69 and production of interferon-γ by a clonal population of CD4+ T cells, increase proportionately with

the addition of increasing concentrations of specific tetramer for staining. Thus, tetramer binding, which indicates a certain level of TCR–MHC–peptide avidity, can itself lead to T cell activation. Other recent studies have compared activation profiles among T cells stimulated with dimers (bivalent class II–peptide complexes), trimers, or tetramers (30–33). These studies indicate that multimerization of TCRs occurs early after interaction with MHC oligomers, followed by rapid T cell activation. Fluorescent staining with the multimers was enhanced by this activation, suggesting that clustering of receptors and/or endocytosis occurs, which augments the fluorescent tetramer signal detection. This relationship between avidity, activation, and tetramer binding is illustrated in Figure 5. Two human T cell clones were used, one of which is activated by low antigen concentration (Figure 5A) and shows a high capacity for fluorescent tetramer binding (Figure 5B). This clone also binds tetramer at both room temperature and 37°C (Figure 5C). In contrast, a low-avidity clone is shown, which requires higher antigen concentrations for stimulation, shows lower fluorescent tetramer binding, and which binds tetramer only at 37°C, not at room temperature (23).

It thus appears that, while high-avidity T cells are readily detected using MHC tetramer fluorescence technology, detection of low-avidity T cells may require simultaneous measurement of activation markers or methods to facilitate enhanced tetramer staining by improving TCR clustering and/or internalization. These studies highlight important issues of T cell frequency, avidity, and activation that have implications for analysis of autoreactive T cells in patients with autoimmune disease.

T cell frequency. Current methodology detects cells that are present in blood at frequencies above ~0.1%, or 1/1,000. With improved methods to lower background staining from non–T cells, this detection threshold may lie closer to 1/2,000. However, this is likely to be below the frequency of autoreactive CD4+ cells circulating in blood, and direct detection of such rare T cells therefore remains elusive. It is reasonable to attempt direct detection strategies in human studies when the antigen challenge is robust, such as in, for instance, vaccine trials, but it is unlikely that this approach will suffice for studies of disease pathogenesis if peripheral blood is the only source of material available for analysis. In vitro amplification of rare antigen-specific T cells, as illustrated in the examples above, is sufficient to raise the numbers of T cells binding tetramers well above the detection threshold, and provides
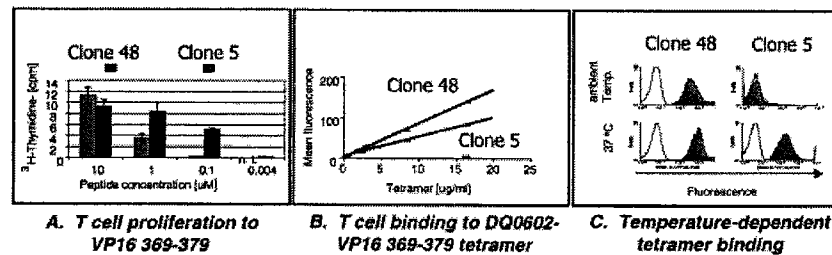
A. *T cell proliferation to VP16 369-379*   **B.** *T cell binding to DQ0602-VP16 369-379 tetramer*   **C.** *Temperature-dependent tetramer binding*

Figure 5. Tetramer binding to CD4+ T cells parallels antigen sensitivity. The relationship between tetramer binding and the avidity of the T cell receptor interaction with major histocompatibility complex–peptide ligands is illustrated, comparing a high-affinity T cell (clone 48) with a moderate-affinity T cell (clone 5). Both T cell clones were derived from an individual (HLA–DQ0602) infected with herpes simplex virus 2, and are specific for the same VP16 peptide, as previously described (23).

direct evidence for the presence of such T cells in the original sample. However, the quantitation of such tetramer-positive cells after in vitro amplification requires the use of a tracking dye such as CFSE in order to calculate the original precursor frequency of positive cells.

TCR avidity. As illustrated above, T cells with high-avidity TCR binding to specific MHC–peptide complexes are readily detected with fluorescent tetramers. Such high-avidity T cells exist in peripheral blood of virally infected or immunized individuals, are sensitive to low concentrations of antigen, and bind tetramers strongly. T cells with low- or moderate-avidity TCR binding also exist in the same individuals.

Avidity of the TCR–MHC–peptide interaction depends not only on the affinity of each TCR for its MHC–peptide ligands, but also on the density and clustering of TCR on the T cell surface. Together with factors such as the density of the MHC on the surface of the antigen-presenting cells, the concentration of peptide, and the type of costimulatory ligands available, TCR signaling then leads to variable outcomes influenced by the strength of the interaction. As discussed below, in clinical studies of patients with autoimmunity, most T cell staining with tetramers analyzed in our laboratories is of low-to-moderate avidity, suggesting either that the specific MHC–peptides used in the tetramers are not the most avid ligands for autoreactive T cells (i.e., we are using the wrong antigens) or, more likely, that circulating autoreactive T cells are indeed of low-to-moderate avidity and that this is intrinsic to their role in immunity.

If the latter premise is correct, detection and monitoring of low-avidity T cells is an important challenge for future clinical applications. Approaches based

on enhancing the clustering and/or internalization of TCR–tetramer complexes are one likely direction, as foreshadowed by the observation that higher temperatures give better staining. It will be worthwhile to search for additional molecular tools to facilitate TCR multimerization on the cell surface and increase the overall avidity of interaction achieved in large complexes.

T cell activation. Another promising direction is to take advantage of the association between tetramer binding and T cell activation. Even in the absence of antigen-presenting cells, soluble tetramers of sufficient avidity can activate T cell clones, as determined by both surface activation markers and cytokine secretion. Thus, analogous to interaction with anti-CD3–activating antibodies such as OKT3, addition of MHC–peptide tetramers to CD4+ T cells can elicit partial activation responses that may be useful as markers for detection of specific cells. One such tetramer-facilitated activation profile is the up-regulation of CD4 on antigen-responsive CD4+ T cells.

This CD4^{high}, CD25+ population has been previously noted to include recently activated T cells, and is a hallmark of in vitro–stimulated antigen-specific CD4+ T cells (25). We have also found these cells frequently among islet antigen-stimulated PBLs of patients with

Table 1. Clinical utility of major histocompatibility complex class II tetramers

Identify antigen-specific T cells
Define intermediate phenotypes involved in disease progression
Distinguish responder versus nonresponder status for different therapeutics
Characterize clinical heterogeneity
Mark T cells for studies of novel genes and molecular pathways
Screen peptide libraries for epitope identification

recent-onset insulin-dependent diabetes mellitus (Reijonen H, et al: unpublished observations). Tetramer analysis of these CD4^{high} activated cells identifies and enumerates the antigen-specific high-affinity cells, which are not present in PBLs from normal controls.

In our initial studies of autoimmune diabetes, the activated, tetramer-positive phenotype was correlated with recent onset of disease, suggesting that detection of these cells may be a useful marker for disease progression and response to therapy. In the rheumatic diseases, similar approaches are feasible, with the limitation that few specific MHC–peptide complexes are known to be important T cell ligands early in the disease. In RA, various studies have implicated such synovial antigens as collagen and glycoprotein 39, but there is little evidence for an important role for T cell expansion to these antigens in disease. In scleroderma, lupus, and many other examples, whether and when circulating CD4+ T cells are critical markers for disease phenotype is debatable.

One rheumatic disease that may be amenable to tetramer approaches is relapsing polychondritis (RP). In this cartilage-specific autoimmune disease, autoantibody responses to type II collagen (CII) are prevalent (34), as are antibodies to other cartilage matrix proteins, including type IX collagen (35) and matrilin-1 (36). CD4+ T cell responses specific to CII are observed in these patients as well (37,38). In RP patients who have shown in vitro responses to CII, initial use of MHC class II tetramers containing CII peptides has produced either low-level, but detectable, staining or specific activation resulting in detection of a CD4^{high}, CD25+ population (39). These findings suggest that this approach could be useful in diseases in which an autoantigen has been identified.

Detection and monitoring of patients' T cell specificity and phenotype are only some of the clinically relevant uses for tetramers. As indicated in Table 1, there are a multitude of applications for this technology in clinical medicine and translational research. In addition, the ability to load the class II α/β dimer with a variety of peptide antigens enables the combination of tetramer analysis with peptide array strategies for epitope identification. We have called this strategy "TGEM," for tetramer-guided epitope mapping, and have reported on its use in identifying T cell epitopes within a complex protein (24). The concept behind the TGEM technique is that a pool of peptides will bind MHC class II molecules competitively, selecting high-avidity binding peptides at the first stage, followed by tetramer staining of human T cells with high-avidity

TCR interactions. In a second-stage analysis, individual peptide–MHC tetramers from the pools that provide positive tetramer staining are used to identify individual epitopes. This approach rapidly and efficiently identifies immunodominant peptide epitopes from complex polypeptide sequences, adding a useful tool for further research into identification of candidate autoantigens.

Summary

Immunotherapies for human autoimmune and immune-mediated diseases are proliferating rapidly, and with these changes comes the opportunity to monitor patients for immune responses to therapy based on early surrogate markers for clinical responses. Class II tetramers have the potential to serve as these sorts of markers for immune monitoring, and thereby assist with patient management, therapy selection, and improved outcomes. However, important issues of TCR avidity require resolution, because much is still unknown regarding location, quantitation, and characterization of the human T cell response. Opportunities for application of tetramer technologies in the near future will enable both clinical progress and the development of new insights into human CD4+ T cell biology in vivo.

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